Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2016; 8(3): 167-171

ISSN- 0975 1556

Research Article

The Effect of *Calotropis gigantea* Leaves Extract on Fibrosarcoma Growth and Caspase 3 Expression.

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Available Online:29th February, 2016

ABSTRACT

The effect of *Calotropis gigantea* (CG) leaves extract on fibrosarcoma growth and Caspase 3 expression have been evaluated. Mile mice were divided into 5 groups with 7 mice per group, (1) Negative control (CMC-Na 0.5%), (2) Positive control (Methotrexate 2,5 mg/kgBW), (3) 50 mg/Kg BW of CG, (4) 100 mg/Kg BW of CG, (5) 150 mg/Kg BW of CG. Mice body weights were observed before induced by cancer agent. Then, all mice were injected by 0.1 mL DMBA 25 µg/0.1 mL acetone by subcutaneous injection two times a week for 6 weeks. After treatment, all mice were sacrificed by euthanasia method based on Guideline for the Euthanasia of Animals. Then, mice nodules were taken and washed with PBS. Nodules were weighted and kept in the formaldehyde solution. Then immunohistochemical caspase-3 specimens were made. *C. gigantea* leaves extract at a dose of 100 and 150 mg/Kg BW were able to inhibit the growth of fibrosarcoma in experimental animals induced DMBA. This extract also improved the apoptotic index of cell, respectively for 20.9 %; 21.5 % and 24.6 %, and increased the caspase-3 expression significantly. Based on those data, *C. gigantea* leaves ethanol extract was potential to develop as anticancer phytopharmaca agent.

Keywords: *Calotropis gigantea*, fibrosarcoma, apoptosis, caspase-3.

INTRODUCTION

Cancer is a leading global cause of death and disability, and responsible for approximately 7.6 million deaths each year[1]. The failure of chemotherapy treatment is due to low selectivity of anticancer drugs on normal cell and cancer cell resistance to chemotherapeutics agents that cause serious side effects in patient. *Calotrophis gigantea* (Apocynaceae) is a traditional medicinal plant that grows widely in Asia (Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, Philippine, Thailand and Srilanka) and Africa (Sudan, Kenya, Tanzania, Gabon, Zaire, Angola, Mozambique, Mauritius, Seychelles)[2,3]. In Indonesia, this plant have been used to cure diseases, e.g. itching, scabies, blain, cough, trachoma, constipation (leaves), asthma, nausea, stomach-ache (flower), gonorrhoea, snake poisoned (root), toothache, swollen, ear inflammation, wormy, and disentry[4].Previous studies reported that *C. gigantea* was potential to be anticancer agent. Cytotoxic activity of cardenolid which was isolated from *C. gigantea* leaves was reported against MCF-7 cancer cell, KB-skin cancer cell, NCL-H18-lung cancer cell[5]; Dichloromethane extract of *C. gigantea* leaves was showed cytotoxic activity against MCF-7 cancer cell, MDA-MB-231 cancer cell, HeLa cell, HT-29 cancer cell, SKOV-3 cancer cell, Hep-G2 cancer cell[6]. Calotropon which was isolated from roots had cytotoxic activity in K562 leukaemia and stomach cancer 790[7]. Methanol extract and chloroform fraction of the root have antitumor activity on *ascites carcinoma*[8]. Anhydrosophoradiol-3-acetate (A3A) isolated from the flower was inhibited the growth of EAC (*Ehrlich Ascites Carcinoma*) effectively[9]. It has been known that cardio glycoside compound isolated from the root was capable to inhibit the growth of HeLa cell and A549 cancer cell[10]. Based on the previous reported data, this research aims to investigate anticancer activity of *C. gigantea* ethanolic leaves extract on fibrosarcoma using experimental animal in vivo, and to determine the mechanism through apoptotic induction with caspase-3 expression as the parameter.

MATERIALS AND METHODS

Plant material

Widuri (*Calotropis gigantea*) was obtained from Lembaga Ilmu Pengetahuan Indonesia (LIPI) Purwodadi, Jawa Timur. Authentication and identification of plant was carried out at Lembaga Ilmu Pengetahuan (LIPI) Purwodadi Jawa Timur.

Animals

Experimental animal for anticancer test was white male mice (*Mus musculus*) furrow Balb/C obtained from Gadjah Mada University, Jogjakarta. The mice had 20 – 30 g body weight and maintained on standard animal pellets and water ad libitum at animal laboratory, Faculty of Science and Technology, Universitas Islam Negeri, Malang. Permission and approval for animal studies were obtained from Faculty of Medicine, Universitas Brawijaya.

Chemicals and reagents

**Author for Correspondence*

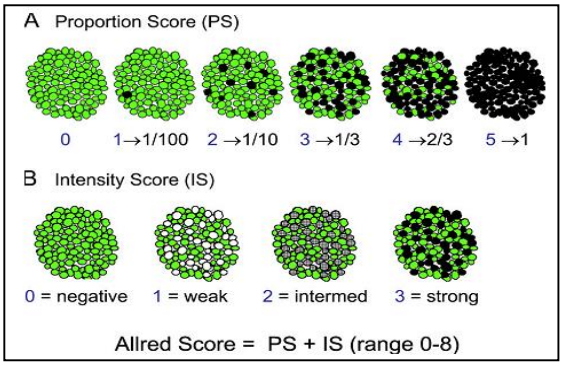

Figure 1: Scoring Allred[13]

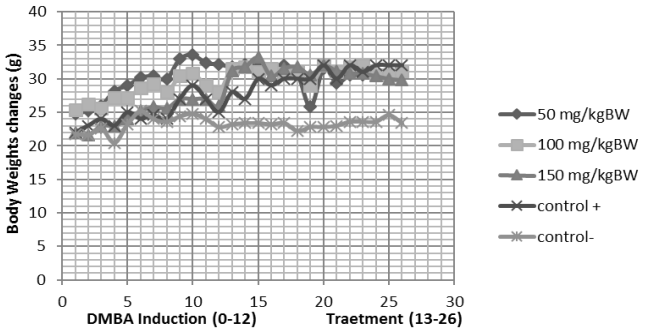

Figure 2: Body weight changes for animals. The mice body weight when induced by DMBA (1-12) and treatment (13-26).

Table 1**:** Results of mice body weight and fibrosarcoma incidence observation.

Group	% Increasing body weight ±SD[1]	Average of tumour weight ±SD[2]	Tumour Incidence
Dosage 50 mg/Kg BW	30.00 ± 2.00	102.3±1.27	40 %
Dosage 100 mg/Kg BW	30.20 ± 2.16	122.05±1.9	20 %
Dosage 150 mg/Kg BW	28.80 ± 1.78	83.3±2.83	20 %
Methotrexate 2.5 mg/Kg BW	27.80 ± 6.14	45.1±1.41	40 %
Negative control CMC-Na 0.5%	22.40 ± 4.88	146.4±3.11	40 %

[1]value showed average of mice body weight ± standard deviation
[2]value showed average of tumour weight ± standard deviation

Methotrexate, 7,12-Dimethylbenz(α) antrasene (DMBA), acetone, CMC-Na, sawdust, paraffin, glycerin, ethanol p.a (100 %, 90 %, 80 %, 70 %), *xylol*, PBS, H_2O_2, primary antibody caspase-3, secondary antibody *Antirabbit* IgG, biotin, SA-HRP (*Sterp Avidin-Horseradish Peroxidase*) (Daco), DAB *substmicee* (Amresco), Meyers Hematoxylin (IHC World).

Plant extraction

Calotropis gigantea leaves were washed and cut into small size, then placed in the oven with temperature of 40 ^oC. Dried leaves were grinded by steamroller 90 meshes. Extraction was using ethanol 70 % as a solvent. Ethanol extract was further concentrated by rotary evaporator and placed in the oven with temperature of 40 ^0C.

Anticancer assay

Total 35 mice were adapted for ± 5 days and divided into 5 groups with 7 mice per group. Mice body weights were observed before induced by cancer agent. Then, all mice were injected by 0.1 mL DMBA 25 µg/0.1 mL acetone by subcutaneous injection two times a week for 6 weeks[11]. The groups were: Negative control group were treated with CMC-Na 0.5% once a day for 14 days. Positive control group were treated with Methotrexate at a dose of 2,5 mg/kgBW once a week for two weeks. First dosage group were treated with *C. gigantea* leaves extract at a dose of 50 mg/kg BW once a day for 14 days. Second dosage group were treated with *C. gigantea* leaves extract at a dose of 100 mg/kg BW once a day for 14 days. Third dosage group were treated with *C. gigantea* leaves extract at a dose of 150 mg/kg BW once a day for 14 days. After treatment, all mice were sacrificed by euthanasia method based on Guideline for the Euthanasia of Animals (by inhalation of ether or chloroform)[12]. Then, mice nodules were taken and washed with PBS. Nodules were weighted and kept in the formaldehyde solution. Then immunohistochemical caspase-3 specimens were made.

Immunohistochemical Caspase-3

Caspase-3 assay was conducted by immunohistochemical colouring standard procedure (10). Caspase-3 polyclonal antibody (rabbit polyclonal anti-mouse caspase-3) (Dako) was used as primary antibody and (anti rabbit biotin conjugated) (Dako) as secondary antibody. Microscopic expression of caspase-3 was observed from the brownish colour that caused by antibody anti caspase-3 reaction visualised by chromogen DAB (Sigma). The assay was done in triplicates. Total expression of caspase-3 was measured by counting the fibroblast cells that showed brownish colour using light microscope (Olympus) at 20 field of view with a 400 times magnification. Apoptotic index was determined by the equation below[13]: $\text{Apoptotic Index (AI)} = \frac{\text{apoptotic cell}}{\text{total cell}} \text{ x } 100\ \%$

Apoptotic index was defined as presentation of apoptotic cell from 1000 cancer cells on 20 fields of view. Apoptotic cell was identified as cell that showed brown colour in its cytoplasm. Analysis also conducted by Allred scoring method to determine the semi quantitative IHK score. IS value was showed intensity score, which were scored as 0 (negative or no brown colour), 1 (weak brown colour), 2 (medium brown colour) and 3 (strong brown colour). IP value was showed the presentation of brown colour, which were scored as 0 (no brown colour), 1 (≤1/100 brown cells), 2 (≤1/10 smeared cells), 3 (≤1/3 smeared cells), 4 (≤2/3 smeared cells), and 5 (all cells are smeared). Total sum of IS value and IP value was total score of IHK[13].

RESULTS AND DISCUSIONS

Induced Mice by DMBA

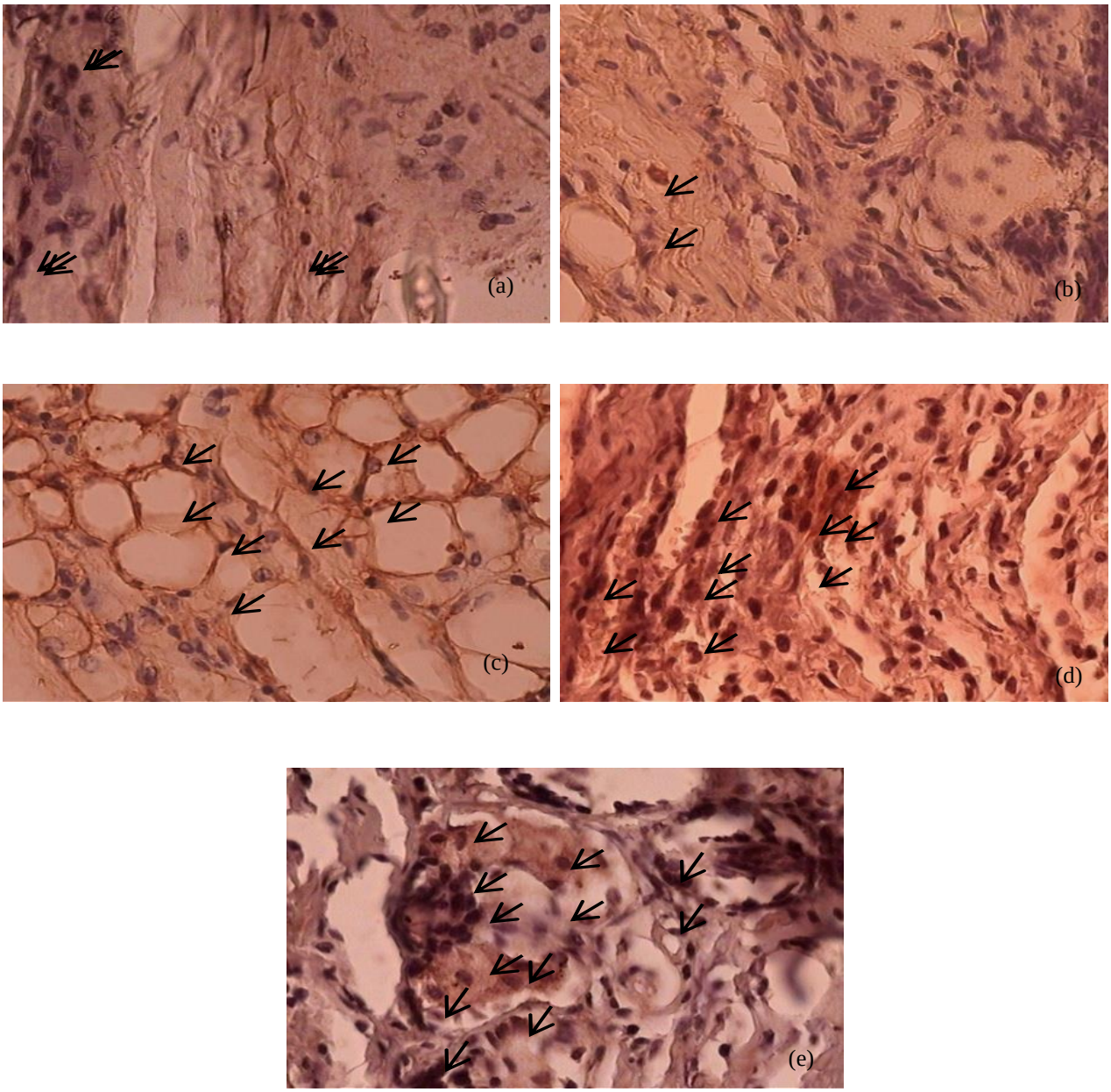

Figure 3: The result of IHK Caspase-3, zoom: 400x, a) negative control, CMC-Na 0.5 %, b) positive control Methotrexate 2.5 mg/Kg BW, c) dosage 50 mg/Kg BW, d) dosage 100 mg/Kg BW, e) dosage 150 mg/Kg BW. Those arrows show the cells that have expression of caspase-3 (apoptotic).

Table 2**:** The analysis result of apoptotic index and IHK caspase-3 score

Group	Live Cell	Death Cell	Apoptotic Index (AI) (%)	Increasing AI (%)	IHK Caspase-3 Score[1]
Negative control	921	79	7.9	0	3.90 ± 0.57
Positive control	819	181	18.1	10.2	4.00 ± 0.47
Dosage 50 mg/Kg BW	712	288	28.8	20.9	4.70 ± 1.06
Dosage 100 mg/Kg BW	707	294	29.4	21.5	5.30 ± 0.82
Dosage 150 mg/KgBW	675	325	32.5	24.6	5.50 ± 0.53

[1] value shows average of IHK caspase-3 score ± standard deviation

Body weight was one kind of respond that showed the growth of the mice since induced by DMBA. Mice body weight observation from 1st until 12th DMBA induction was shown an increase and decrease in unstable. In the DMBA induction at 4th, 8th, 11th and 12th, the average of mice body weight in all treatment groups experienced a decline. Mean, when extract treatment was conducted, the mice body weight tend to be more stable at 13th until 36th

measurement. Stability of mice body weight possibly caused by pains less and lack of stress because of termination of DMBA induction during treatment period. The mice body weight was increased since first until last treatment (figure 2). Statistical analysis showed that treatment had influenced on mice body weight. The result of One Way ANOVA assay was p = 0.026 (p<0.05), it showed that there was a significant difference in all treatment groups. The Post Hoc analysis result showed that there was significant difference between group at a dose of 50 mg/Kg BW and negative control group with p = 0.037 (p<0.05), then group at a dose of 100 mg/Kg BW and negative control group with p = 0.031 (p<0.05). Treatment group with *C. gigantea* leaves extract at a dose of 100 and 150 mg/Kg BW showed lower fibrosarcoma insidence than negative control group. *C. gigantea* leaves extract at a dose of 100 and 150 mg/Kg BW were capable to diminish fibrosarcoma incidence and inhibited fibrosarcoma growth**.** The result was equal to tumour weight that formed after treatment. When *C. gigantea* leaves extract dosage was increased, the tumour weight was decreased.

Immunohistochemical (IHK) Caspase-3

IHK analysis was used to know the apoptotic cell as the parameter of body immune system reaction in inhibited the growth of cancer. The principle of IHK analysis was antibody bonded antigen specifically. Antibody-antigen bonding was formed between conjugated antibody and peroxidase (catalytic enzyme that can give specific colour). This antibody-antigen bonding can be located in the cytoplasm or in the cell core that was showed by brown colour[14]. Based on analysis of index apoptotic, it indicated that increasing the dosage of *C. gigantea* leaves extract can increase the apoptotic index. The result of apoptotic analysis and IHK caspase-3 score was presented on table 1. The result of Kruskal-Wallis assay showed 'p' value 0.000 (p<0.05), it means that there was a significant difference between treatment groups. Then the next analysis is Mann-Whitney assay, and IHK score from this assay in treatment group with dosage 50, 100, and 150 mg/Kg BW (p value <0.05) shows that there was a significant difference to the negative control group. This result indicated that *C. gigantea* leavesextract able to increase caspase-3 expression at induced mice by DMBA. Result of this caspase-3 score was correlated with increasing of apoptotic index and decreasing tumour weight. Inhibition of fibrosarcoma growth and increasing of caspase-3 expression caused by *C. gigantea* leaves extract treatment were suspected because of cardiac glycosides (CGs) in this extract. CGs compound that isolated from *C. Gigantea* leaves were reported, including 19-Nor- and 18,20-Epoxy-cardenolides, calotropin, calotropogenin[5]. Based on in vivo study, CGs compound known had high anticancer activity on some types of breast cancer, lung cancer, prostate, melanoma, neuroblastoma, myeloma, and leukemia[15]. And also showed the inhibition on skin carcinogenesis, lung cancer and decrease tumour weight[16-18]. Based on clinical test, cardiac glycosides were proved to have anticancer activity at breast cancer patient[18]. The mechanism of apoptotic induction and cell proliferation inhibition of CGs compound were known from alteration pathway in homeostasis of K^+, Na^+ and Ca^{2+}, induction of mitochondrial pathway and inhibition of TNF/NF-κβ[18,19].

CONCLUSION

C. gigantea leaves extract at a dose of 100 and 150 mg/Kg BW were able to inhibit the growth of fibrosarcoma in experimental animals induced DMBA. This extract also improved the apoptotic index of cell, respectively for 20.9 %; 21.5 % and 24.6 %, and increased the caspase-3 expression significantly. Based on those data, *C. gigantea* leaves ethanol extract was potential to develop as anticancer phytopharmaca agent.

ACKNOWLEDGMENT

The author would like to acknowledge the assistance of this research funds to The Directorate of Islamic Higher Education Ministry of Religious Indonesia through a competitive grant programme Science and Technology (Program Hibah Bersaing Sains dan Teknologi; PST) 2015.

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